Biological Control 46 (2008) 542–546

Contents lists available at ScienceDirect

Biological Control

journal homepage: www.elsevier.com/locate/ybcon

Genetic and biological comparisons of four nucleopolyhedrovirus isolates that are infectious to *Adoxophyes honmai* (Lepidoptera: Tortricidae)

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ARTICLE INFO

Article history:
Received 24 March 2008
Accepted 9 June 2008
Available online 15 June 2008

Keywords:
Adoxophyes honmai
Adoxophyes orana
Baculovirus
Lethal dose
Nucleopolyhedrovirus
Restriction endonuclease analysis
Speed of kill
Virus yield

ABSTRACT

The smaller tea tortrix, *Adoxophyes honmai* (Lepidoptera: Tortricidae), is one of the most important pests of tea plants in Japan. *Adoxophyes honmai* nucleopolyhedrovirus (AdhoNPV) isolates from Tsukuba (AdhoNPV-Ts) and Tokyo (AdhoNPV-To), Japan, and *Adoxophyes orana* nucleopolyhedrovirus (AdorNPV) isolates from England (AdorNPV-En) and the Netherlands (AdorNPV-Ne) were subjected to genetic and biological comparisons to select a candidate NPV isolate to control *A. honmai*. Restriction endonuclease (REN) analysis demonstrated that AdhoNPV-Ts and AdhoNPV-To had similar REN patterns, whereas AdorNPV-En and AdorNPV-Ne exhibited different REN patterns from each other as well as those of AdhoNPV-Ts and AdhoNPV-To. Bioassays with fourth-instar *A. honmai* larvae showed that AdorNPV-En was most pathogenic, with the lowest LD_{50} of 37 occlusion bodies (OBs) per larva. When *A. honmai* neonates were inoculated with each isolate, most larvae infected with AdhoNPV-Ts and AdhoNPV-To were killed in the final (fifth)-instar, whereas larvae infected with AdorNPV-Ne were killed at every instar and larvae infected with AdorNPV-En were killed at the first- to third-instar. AdorNPV-En or AdhoNPV-Ts fed to neonates had the shortest or longest killing times, respectively, with ST_{50} values of 6 and 19 days. AdhoNPV-To and AdorNPV-Ne had intermediate killing times. The OB yield per larva of AdhoNPV-Ts and AdhoNPV-To was significantly higher than that of AdorNPV-En and AdorNPV-Ne. Our results suggest that AdorNPV-En is suitable as an inundative agent because it is a quick-killing, highly virulent NPV, and AdhoNPV-Ts and AdhoNPV-To are more appropriately used as inoculative agents because of their high OB production.

1. Introduction

Baculoviruses are safe and selective bioinsecticides that are restricted to invertebrates (Moscardi, 1999). Baculoviruses comprised of nucleopolyhedroviruses (NPVs) and granuloviruses (GVs) are expected to play an important role in integrated pest management (IPM). Basic approaches for the use of insect pathogens, including baculoviruses, as microbial control agents are classical biological control, inoculative biological control, inundative biological control, and conservation and enhancement of natural enemies (Hajek, 2004). To use baculoviruses efficiently as control agents, it is necessary to identify the pathological characteristics of candidate viruses to select appropriate management approaches.

The smaller tea tortrix, *Adoxophyes honmai* Yasuda (Lepidoptera: Tortricidae), is one of the most important pests of tea plants in Japan. It overwinters in the middle larval stage, has five larval instars, pupates, emerges as an adults, and generally has four generations a year, but an additional generation is often observed in the southern part of Japan (Tamaki, 1991).

Along with *A. honmai*, another key pest of tea is the oriental tea tortrix, *Homona magnanima* Diakonoff (Tamaki, 1991). Both tortricids are susceptible to GV infection and GVs of *Adoxophyes orana* Walsingham (AdorGV) and *H. magnanima* (HomaGV) have been used to control *A. honmai* and *H. magnanima* larvae in Japanese tea fields (Kunimi, 1998, 2007; Tamaki, 1991). A commercial product, Hamaki-Tenteki® (Arysta LifeScience Corp., Tokyo, Japan) which contains AdorGV and HomaGV, was registered as a biopesticide in 2003 in Japan (Kunimi, 2007). Hamaki-Tenteki® is an effective control agent against *A. honmai* and *H. magnanima* but has a slow killing action; it takes approximately 40 days to kill *A. honmai* neonates after inoculation (Yamada and Oho, 1973; Nakai and Kunimi, 1997). Because of the slow killing action of the AdorGV in the commercial product, *A. honmai* larvae continue to feed and damage the tea leaves. Therefore, a pathogen with a shorter period of lethal infection is needed.

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1049-9644/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.biocontrol.2008.06.003

NPVs that infect *A. honmai* have been isolated from Japan and some European countries (Nakai et al., 1997; Ishii et al., 2003). NPVs are attractive control agents because the killing time of NPVs is generally shorter than that of GVs. Thus, these NPV isolates might have better pathological characteristics as microbial agents than Hamaki-Tenteki®.

In this study, we characterized four NPV isolates by restriction endonuclease (REN) analysis of viral DNA, compared the responses of first- and fourth-instar *A. honmai* larvae to these NPV isolates, and discussed the potential use of these NPV isolates to control *A. honmai* larvae.

2. Materials and methods

2.1. Insects

Adoxophyes honmai used in this study were initially supplied as eggs by Agro-Kanesho Co. Ltd. (Saitama, Japan). Insect colonies were maintained continuously in the laboratory, and all experiments were conducted at 25 °C with a 16-h photoperiod (L16:D8). Larvae were reared on an artificial diet (Silkmate 2S; Nihon Nosan-Kogyo Co. Ltd., Yokohama, Kanagawa, Japan).

2.2. Viruses

Two isolates of *A. honmai* NPV (AdhoNPV) were from Japan with the Tsukuba (AdhoNPV-Ts) and Tokyo (AdhoNPV-To) isolates obtained from diseased larvae collected in tea fields in Tsukuba, Ibaraki (Nakai et al., 1997, 2003), and Mizuho, Tokyo, respectively. Two isolates of *A. orana* NPV (AdorNPV) were obtained from diseased *A. orana* larvae collected in England (AdorNPV-En) and the Netherlands (AdorNPV-Ne). AdorNPV-Ne was provided by Dr. M.B. Ponsen, Wageningen Agricultural University and was identical to the *A. reticulana* Hübner NPV used by Ponsen and Jong (1964). These isolates were propagated in laboratory cultures of *A. honmai* larvae. Occlusion bodies (OBs) of each NPV isolate were purified basically as described by Ishii et al. (2002). The concentration of OBs in the stock suspension was determined using a bacterial counter (Erma, Tokyo, Japan) under a phase-contrast microscope at 600x. Purified OBs were stored at 4 °C until used.

2.3. Isolation and REN analysis of viral DNA

Purified OB suspension was incubated with a one-third volume of 0.3 M Na_2CO_3 and 0.5 M NaCl at 37 °C for 30 min to dissolve the polyhedrin matrix. Undissolved OBs and heavy debris were pelleted by centrifugation at 1500*g* for 5 min. The supernatant was transferred to a sterile microcentrifuge tube and centrifuged at 20,400*g* for 15 min. The pellet was suspended in 300 μl of cell lysis solution (10 mM Tris and 100 mM EDTA, 1% SDS, pH 8.0) with 1.5 μl of Proteinase K solution (20 mg/ml), and then incubated at 55 °C for 3 h. Viral DNA was extracted using a Puregene DNA Purification Kit (Gentra Systems, Minneapolis, MN, USA) following the manufacturer's instructions.

For REN analysis, 1 μg of viral DNA was digested with 10 U of restriction enzyme [EcoRI, PstI (Takara Shuzo Co. Ltd., Kyoto, Japan), or HindIII (Toyobo Co. Ltd., Tokyo, Japan)] at 37 °C for 4 h, as recommended by the supplier. Digested fragments were separated with the addition of a one-sixth volume of 6× loading buffer (1 mM EDTA, 0.25% bromophenol blue, 30% glycerol) in 0.6% agarose gel using Loading Quick λ/EcoRI digest–λ/HindIII digest (Toyobo Co. Ltd.) as a molecular size marker. The gels were stained with ethidium bromide and photographed under a UV transilluminator using the DIANA II Chemiluminescence Detection System (Raytest Isotopenmeßgeräte GmbH, Straubenhardt, Germany).

2.4. Bioassay

Neonates and fourth-instar larvae of *A. honmai* were bioassayed using a modified droplet feeding method (Kunimi and Fuxa, 1996). Third-instar larvae that were beginning to molt, as determined by head capsule slippage, were transferred to plastic containers (15 cm in diameter, 9 cm in height) without food; newly molted insects were collected after 24 h. Neonates and newly molted fourth-instar larvae were allowed to feed on droplets of viral suspension containing 10% sucrose and 5% red food coloring. As a control, larvae were fed droplets of the same solution without virus. Larvae that ingested the suspension were immediately transferred into 20-ml cups containing fresh artificial diet (INSECTA LF; Nihon Nosan-Kogyo Co. Ltd.). Four or five appropriate concentrations (10^5, $10^{5.5}$, 10^6, $10^{6.5}$, 10^7, $10^{7.5}$, or 10^8 OBs/ml) were selected for neonates and four or five appropriate concentrations (10^4, $10^{4.5}$, 10^5, $10^{5.5}$, 10^6, $10^{6.5}$, or 10^7 OBs/ml) were selected for fourth-instar larvae. The mean volume ingested by neonates and fourth-instar larvae was 8.2 ± 1.6 and 480 ± 1.6 nl (mean ± SE), respectively (Ishii et al., 2003); therefore, LC_{50} could be converted by simple mathematics to LD_{50}. The experiments were replicated twice with 30–40 larvae/dose/replication. Larvae were reared until they died or pupated. Tissue smears prepared from dead insects were examined for OBs under a phase-contrast microscope at 600x.

2.5. Speed of kill

Adoxophyes honmai neonates were inoculated with the LD_{90} of each NPV isolate or no virus by the droplet feeding method as described in Section 2.4., and were transferred individually into 20-ml cups containing fresh artificial diet. The larvae were checked daily for mortality; the developmental stage of larvae and developmental stage at death were recorded. Thirty to 38 larvae were used for each experiment.

2.6. Yield of OBs

Neonates and fourth-instar larvae were inoculated with the LD_{90} of each NPV isolate or were fed no virus by the droplet feeding method, and were transferred individually into 20-ml cups containing fresh artificial diet and remained there until death or pupation. Dead larvae were weighed, individually transferred into a 1.5-ml microcentrifuge tube, and stored at −35 °C until used. Eight to 10 NPV-killed larvae were randomly selected from each group for OB counting. Each infected larva was homogenized in 300 μl of PBS with 0.05% Tween-20. The number of OBs in each sample was determined using a bacterial counter under a phase-contrast microscope. The OB counts were replicated three times for each sample.

2.7. Data analysis

Mortality caused by infection with each NPV isolate was evaluated by probit analysis (Finney, 1971) in POLO-PC (LeOra Software, Berkeley, CA). The Kaplan–Meier product limit estimator was used to estimate the median survival time (ST_{50}) for each treatment using JMP 4 (SAS Institute Inc., Cary, NC). OB yield was log-transformed and analyzed using a one-way analysis of variance (ANOVA) followed by Tukey–Kramer multiple comparison tests in JMP.

3. Results and discussion

3.1. REN analysis of viral DNA

The REN analysis using HindIII showed that the four NPV isolates had different REN profiles (Fig. 1). REN analyses using EcoRI